Supporting Information

Real-time Ratiometric Imaging of Micelles Assembly State in a Microfluidic Cancer-on-a-chip

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Supporting Videos

The videos demonstrate real-time imaging during perfusion of lateral channel (blood vessel model channel) of the microfluidic cancer-on-a-chip model. In each case hybrids are perfused through left-hand side channel (left from the triangular posts) and further retained or not from penetration of the right-hand side channel (the ECM model)

Supplementary Video S1 Perfusion of hybrid 1 into the healthy blood vessel model channel.

Supplementary Video S2 Continuation of perfusion of hybrid 1 into the healthy blood vessel model channel (continuation of video 01a), demonstrating retention of the perfused polymer by the HUVECs barrier.

Supplementary Video S3 Perfusion of hybrid 1 into the tumor blood vessel model channel, demonstrating impaired retention of HUVECs barrier.

Supplementary Video S4 Ratiometric video of perfusion of hybrid 1 into the tumor blood vessel model channel, demonstrating real-time imaging and detection of the hybrid's assembly state.

Supplementary Video S5 Perfusion of hybrid 1 into non-endothelialized lateral channel, demonstrating instant penetration of the hybrid into the collagen gel present in the central channel.

Supporting Figures

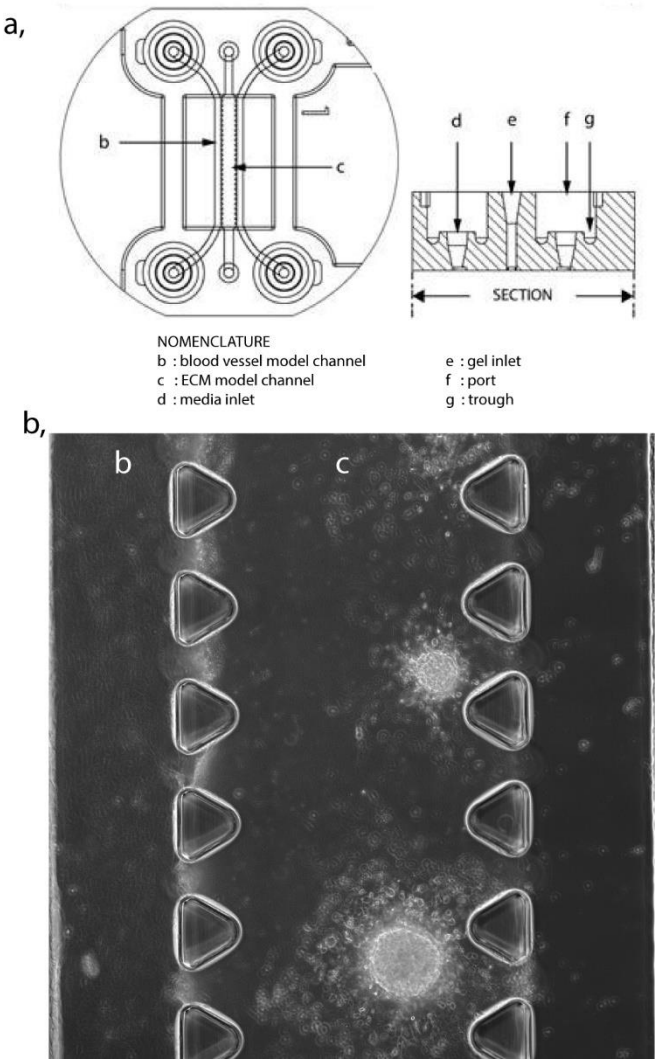

Figure S1. a, Microfluidic chip used to recreate cancer-on-a-chip model. It consists of three microfluidic channels: the central channel with 1.3 x 0.25 mm (w x h) and the two media channels with 0.5 x 0.25 mm (w x h) dimensions. The middle channel is separated from the two lateral

channels by rows of triangular posts distant by 100 μm from one another. **b,** transmission image of recreated cancer-on-a-chip model with HUVECs lining the blood vessel model channel (b) and collagen gel embedded HeLA spheroids in ECM model channel (c)

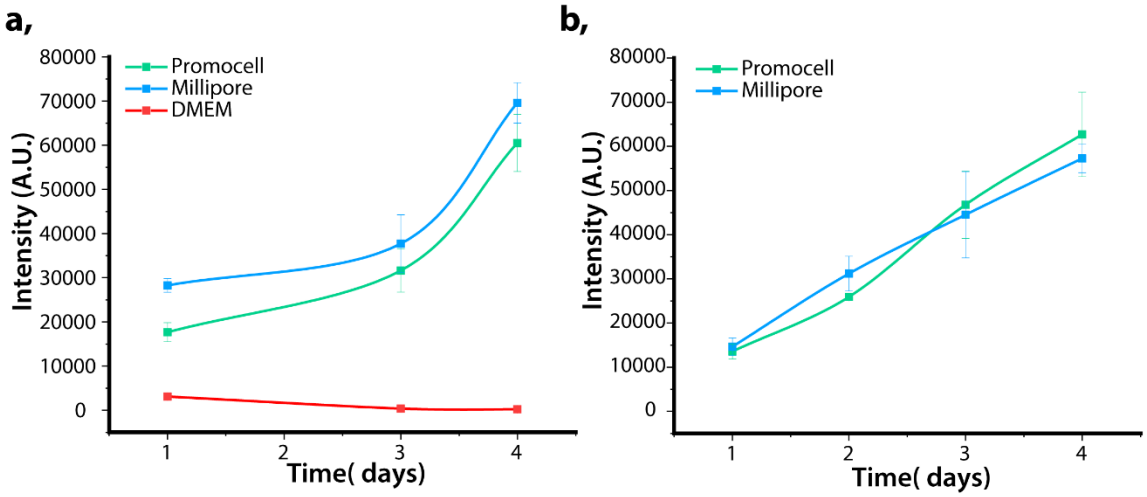

Figure S2. Growth kinetics of HUVECs and HeLa. **a,** HUVECs were seeded in a 96 well plate at a density of 2500 cells/well and incubated with three different media types: DMEM medium supplemented with 10% FBS (which is used for HeLa cell monoculture) and Promocell or Millipore media optimized for HUVECs. PrestoBlue cell viability test was performed after 1, 3 and 4 days of incubation. The absorbance at 570 nm is plotted as a function of time. HUVECs did not grow in DMEM medium but they grew similarly using both HUVEC optimized media. **b,** HeLa cells were seeded at a density of 2500 cells/well in a 96 well plate and incubated with the two HUVEC media to decide which was the optimal for HeLa grow. PrestoBlue cells viability test was performed after 1, 3 and 4 days. The graph shows the absorbance intensity as a function of time. HeLa cells growth kinetic was similar using both media. Every condition was performed in sextuplicate, error bar represents S.D. between wells.

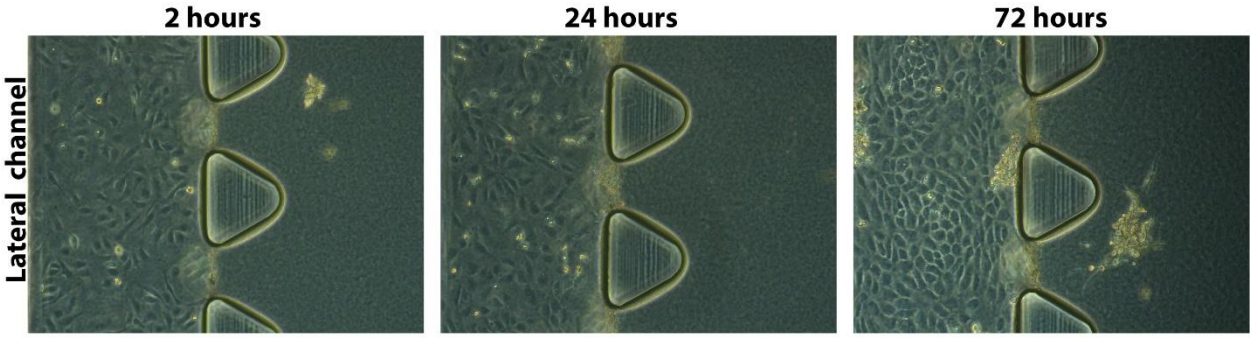

Figure S3. HUVECs monolayer formation. Cell growth in the microfluidic chip after 2 hours, 24 hours and 72 hours. Magnified image of the lateral channels (left hand side from the triangular posts) where HUVECs were seeded to form the blood vessel model and part of the gel channel (right hand side from the posts). Distance between triangular posts is 100 μm.

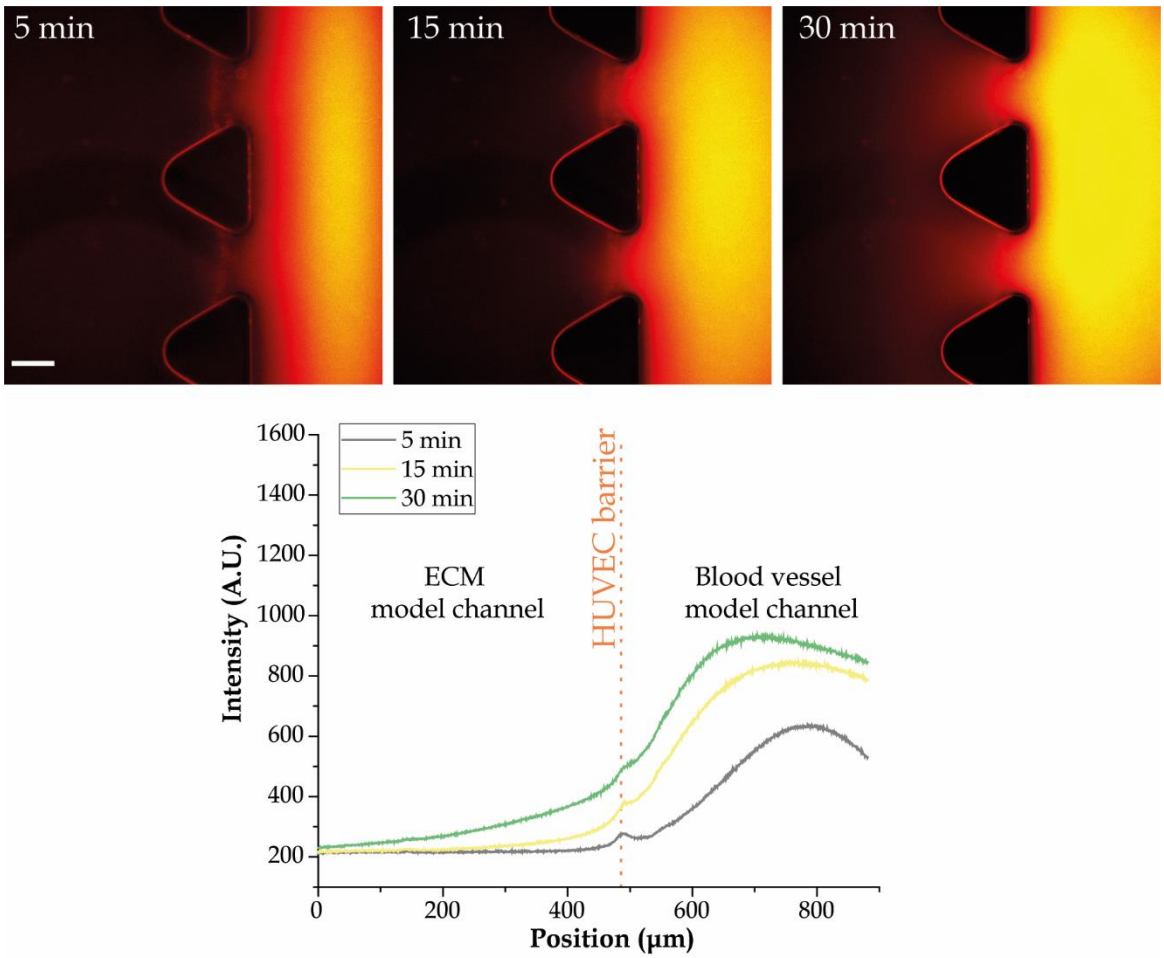

Figure S4. Snapshots of continuous perfusion of 10 kDa dextran through blood vessel model channel. 3 time points have been selected to show the HUVECs barrier functionality, from left to right: 5 minutes, 15 minutes and 30 minutes. Scale bar 100 µm. The graph below quantifies the mean fluorescent intensity of the image in each position, we can see that at time 5 minutes the dextran did almost not cross trough the HUVECs barrier, while in time some of the molecules cross, still the intensity in the ECM model channel is much lower than the one of the blood vessel model perfused channel.

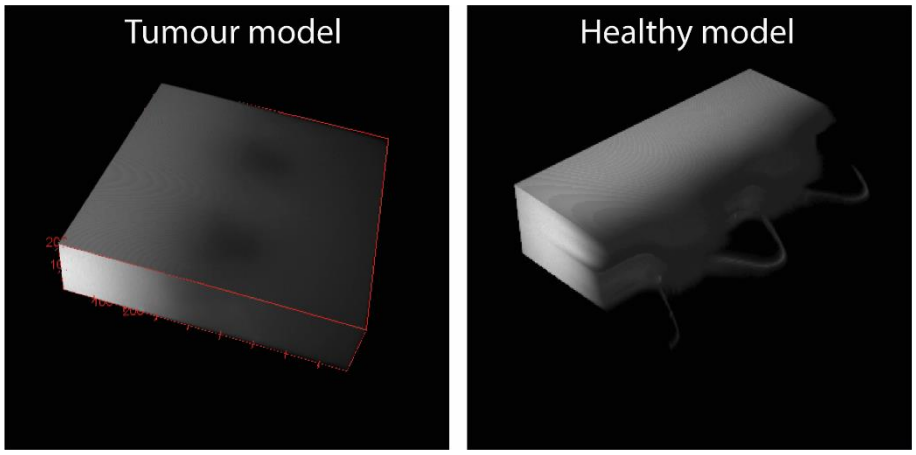

Figure S5. Epifluorescent microscopy 3D reconstructed image of continuously perfused 10 kDa Dextran through the endothelialized blood vessel model channel (as in S4) in a cancer model chip (endothelialized blood vessel model channel and HeLa spheroids embedded into the gel matrix, left) compared to a healthy model (right) to demonstrate the permeability and retention, respectively.

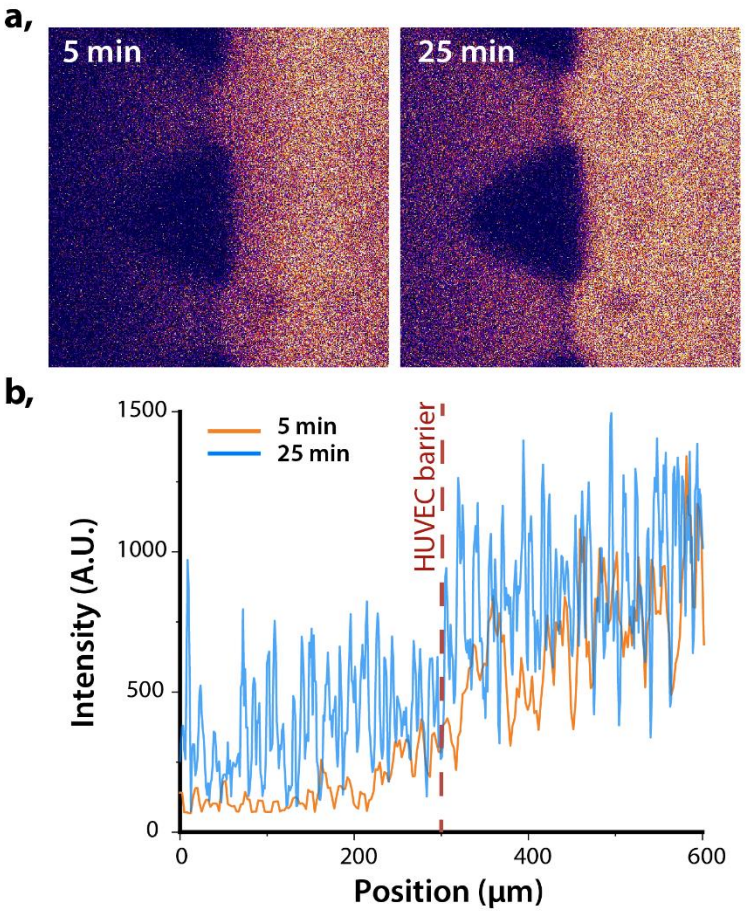

Figure S6. Extravasation of hybrid 4 (6-7 kDa) in healthy model already occurs after 5 minutes of continuous perfusion. A healthy blood vessel model was prepared and hybrid 4 was flow for 25 minutes to study its extravasation **a.** Confocal images of the hybrid 4 being flow through the blood vessel model channel at 5 and 25 minutes. In the images the perfused channel (right) and part of the ECM model (left) can be observed. Look-up table "fire" is used for visualization purposes **b.** Plot profile of a horizontal line across each image showing the intensity detected in each position. After 5 minutes of perfusion the hybrid was detected in both: the gel and the lateral channel, however the intensity in the gel channel was only 15% comparing to the blood vessel model channel, while after 25 minutes it raised to 40%.

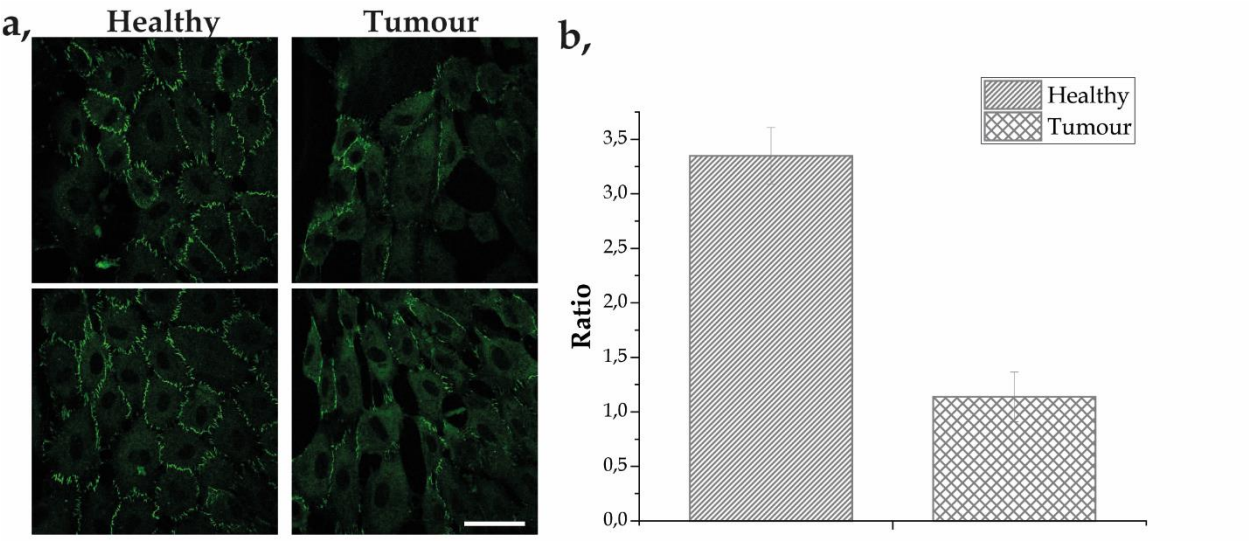

Figure S7. a, Confocal images of ZO-1 expression in the HUVECs monolayer lining the healthy and cancer blood vessel model channel. Scale bar 50 μm. b, Ratio of ZO-1 expression in the membrane of the cells (tight junction formation) compared to the intracellular expression. 5 different images of 2 different chips were analyzed, where 6 membrane ROIs and 6 intracellular ROIs were defined. The mean intensity of the 6 ROIs of the membrane was divided to the mean intensity of the intracellular ROIs. The graph shows the mean ratio ± s.d. of the 5 analyzed images.

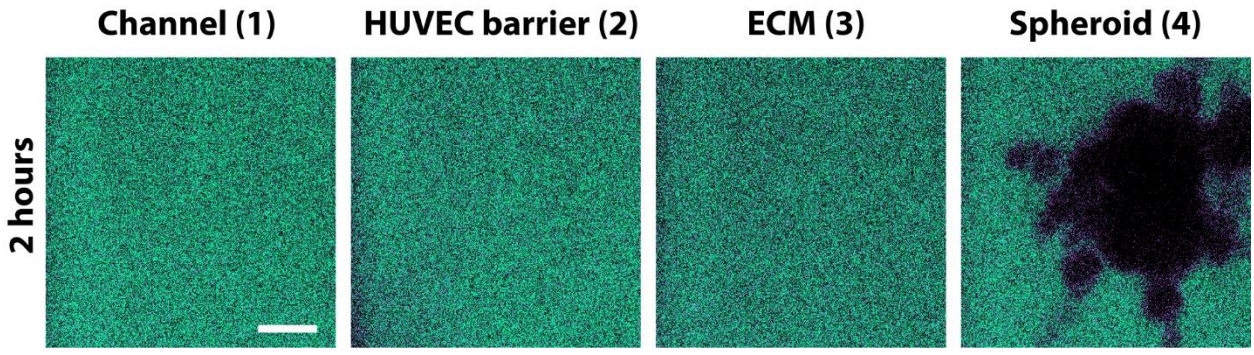

Figure S8. Space resolved stability of hybrid **1** after 2 hours of continuous perfusion through the blood vessel model channel. Ratiometric confocal images of real-time monitored presence of

micelle (green) and monomer (magenta) at reconstructed barriers (1 - blood vessel model channel, 2 - HUVECs barrier, 3 - ECM and 4 - HeLa Spheroid). Scale bar 20μm.

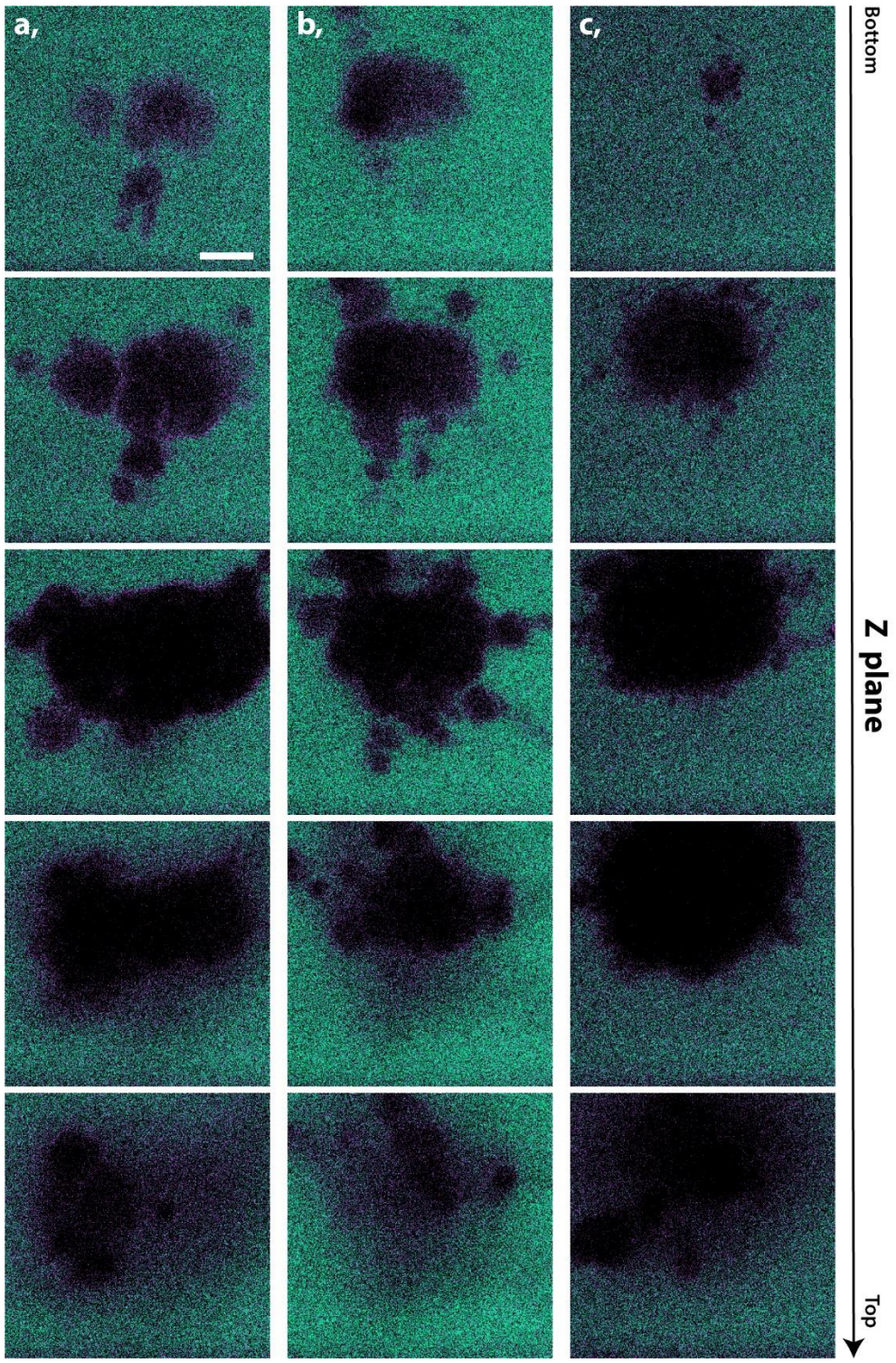

Figure S9. Penetration and stability of hybrid 1 in the HeLa spheroids after 2 hours of continuous perfusion throught the blood vessel model channel. Ratiometric confocal images of real-time

monitored presence of micelle (green) and monomer (magenta). **a.** Z-stack images of spheroid 1 **b.** Z-stack images of **s**pheroid 2 **c.** Z-stack images of **s**pheroid 3. Scale bar 20µm

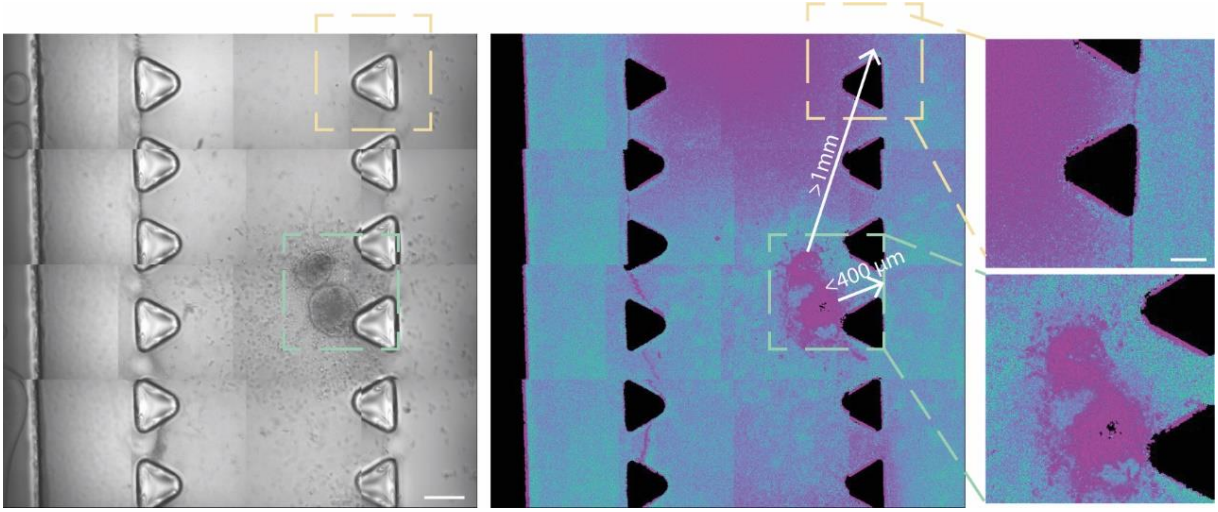

Figure S10. Transmission images of cancer model chip and corresponding ratiometric image, describing "far" and "close" selection criteria, referring to the distance of HUVECs barrier to the nearest gel embedded HeLa spheroid. Scale bar stitched image 200 µm, scale bar zoomed in image: 100 µm. If HeLa spheroids are closer than 400 µm we considered the region as "close" to HeLa. In contrast, if HeLa spheroids were at a distance larger than 1 mm we considered the region as "far" from HeLa. Yellow (upper) dashed squared area demonstrates studied EB in "far" zone, and green (lower) dashed area represents studied EB in "close" zone.

Supporting Discussion

The preparation of tumor model chip consisted on a procedure described in the Materials and Methods section of the article. Despite the followed protocol was in all experiments the same, each

chip preparation presented few differences, also reflected when infusing hybrids. Those differences relate mostly to the number, and distribution of spheroids within the extracellular matrix (collagen gel). Upon chip preparation we could adapt the number of spheroids from few to over a dozen and adjust the number accordingly through a visual inspection. However, the distribution of spheroids across the ECM can be hardly controlled, especially their distance from the endothelial barrier. Within the performed experiments (infusion of hybrids into the blood vessel model channel) we observed certain heterogeneity in the retention of perfused material in the HUVECs lined channel. We registered either immediate monomer/micelle crossing the EB or more gradual, depending on the regions of the microfluidic chip. Overall, the immediate passage of the perfused polymer was attributed to the HeLa spheroids present very closely to the EB (few to 400 μm away), in such cases the hybrids entered the gel channel in less than a minute. On the other hand, when the spheroids were located further away (more than in the radius of 1 mm) the HUVEC wall appeared leaky, but the rate of perfused structures entering the ECM was variable. Depending on the tested chip, the detected fluorescence intensity was similar on both sides of the HUVECs wall (in the blood vessel model and ECM model channels) within few to tens of minutes and in some areas even after 30 minutes. As reported in literature, the coculture with HeLa affects the endothelial cells, not only through cell-cell contact (when we observed very rapid entering of the hybrids into the ECM), but also via paracrine communication. We hypothesize that this transmission of information via culture medium cannot be easily controlled in dynamic conditions and 3D spheroid random distribution, as it could be in a static setup, where the system is allowed to reach an equilibrium. As the HeLa-excreted molecules are harmful to HUVECs, their integrity becomes impaired and the effect likely depends on the exposure to these damaging species.

Overall, this heterogenous leakiness of HUVECs barrier reflects the features of EPR heterogeneity observed *in vivo*, which is highly dependent on cancer type, stage and patient.